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The calpain–calpastatin system and protein degradation in fusing myoblasts

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Abstract

Calpain (Ca^{2+}-activated cysteine protease) induced proteolysis has been suggested to play a role in myoblast fusion. We previously found that calpastatin (the endogenous inhibitor of calpain) diminishes markedly in myoblasts during myoblast differentiation just prior to the start of fusion, allowing Ca^{2+}-induced calpain activation at that stage. Here, we show that a limited degradation of some proteins occurs within the myoblasts undergoing fusion, but not in proliferating myoblasts. The protein degradation is observed at the stage when calpastatin is low. Protein degradation within the myoblasts and myoblast fusion are inhibited by EGTA, by the cysteine protease inhibitors calpeptin and E-64d and by calpastatin. The degradation appears to be selective for certain myoblast proteins. Integrin $\beta 1$ subunit, talin and β-tropomyosin are degraded in the fusing myoblasts, whereas α-actinin, β-tubulin and α-tropomyosin are not. A similar pattern of degradation is observed in lysates of proliferating myoblasts when Ca^{2+} and excess calpain are added, a degradation that is inhibited by calpastatin. The results support the notion that degradation of certain proteins is required for myoblast fusion and that calpain participates in the fusion-associated protein degradation. Participation of calpain is made possible by a change in calpain/calpastatin ratio, i.e., by a diminution in calpastatin level from a high level in the proliferating myoblasts to a low level in the differentiating myoblasts. Degradation of certain proteins, known to be responsible for the stability of the membrane–skeleton organization and for the interaction of the cell with the extracellular matrix, would allow destabilization of the membrane and the creation of membrane fusion-potent regions. © 1998 Elsevier Science B.V.

Keywords: Myoblast fusion; Myoblast protein degradation; Calpain; Calpastatin

1. Introduction

During development, myoblasts cease to divide, differentiate and fuse to form multinucleated muscle cells. Myoblast differentiation and fusion to multinucleated myotubes can be achieved in myoblast cultures [1,2]. Myoblast cultures provide a convenient system for studying some of the steps and factors involved in muscle formation, with implications for cell membrane fusion processes in general. Studies on the morphological aspects of cell fusion have delineated multiple steps in a variety of fusion processes, involving cell–cell alignment, tight apposition of the cell membranes and formation of particle-free, phospholipid-rich membrane domains [3–9]. The ac-

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tual fusion first involves small areas, where the breakdown of the apposed membranes occurs, with the establishment of small regions of cytoplasmic continuity between the fusing cells [3–9].

Proliferating myoblasts have well organized membrane and skeletal framework, which become temporarily destabilized in differentiating, fusing myoblasts [7]. It has been proposed that membrane protein degradation contributes to the disorganization of cell membrane and cytoskeleton in fusing cells, including fusing myoblasts, and that calpain (intracellular, Ca^{2+}-dependent cysteine protease) is involved in the fusion-associated protein degradation [10–15]. Myoblast fusion has been shown to be inhibited by cell penetrating cysteine protease inhibitors [13–17].

Calpain is present in a wide variety of cells, along with its endogenous inhibitor, calpastatin [18–20]. Using red cells as models for cell membrane fusion, we previously found that calpain-induced degradation of certain membrane proteins was a prerequisite for red cell membrane fusion and that the cell fusibility depended on the ratio of the red cell calpain to calpastatin [21,22]. We found that in rat L8 myoblasts, the level of calpain (as estimated by antibodies) did not change during myoblast differentiation and fusion, but that a transient diminution of calpastatin occurred in fusing myoblasts [16]. Using Ca^{2+} and the artificial substrate casein in a standard assay for the estimation of calpain activity, we observed degradation of casein when it was added to extracts of fusing myoblasts at the stage when calpastatin level was low, but not when added to extracts of proliferating cells [16]. These results indicated that the diminution in calpastatin allowed the activation of calpain in the fusing myoblasts [16].

In order to find out whether degradation of endogenous proteins occurs within these myoblasts, we studied fusing and non-fusing myoblasts, using antibodies to several membrane and cytoskeletal proteins. Here we show that a limited degradation of some membrane and cytoskeletal proteins occurs within myoblasts undergoing fusion. The endogenous protein degradation is observed at the stage when calpastatin is low and is inhibited by calpain inhibitors. The degradation appears to be selective for certain myoblast proteins. A similar pattern of degradation is observed in lysates of proliferating myoblasts when Ca^{2+} and excess calpain are added, a degradation that is inhibited by calpastatin. The overall results support the notion that a degradation of certain cytoskeletal and membrane proteins participates in the events which lead to destabilization of the membrane and the creation of membrane fusion-potent regions and that calpain is involved in the fusion-associated protein degradation.

2. Materials and methods

2.1. Myoblast cultures

Rat myoblast L8 cell line was obtained from Dr. D. Yaffe (The Weizmann Institute of Science, Rehovot, Israel). The cells were cultured and induced to differentiate, as described previously [17]. Briefly, myoblasts were grown in Waymouth medium in the presence of 15% F.C.S., 1% antibiotics and 1% glutamine (growth medium). When the number of cells reached about 50% confluency, the medium was changed to DMEM, supplemented by 2% horse serum, 1% antibiotics, 1% glutamine and four units of insulin/100 ml medium (fusion medium). The fusion medium was replaced every 48 h. Inhibitors used were EGTA, E-64d (Sigma, USA), calpeptin (Ref. [14], a gift from Dr. T. Tsujinaka, Osaka University Medical School, Suita, Osaka, Japan) and human recombinant calpastatin (M.W. 14 000, Calbiochem, USA). Each of the reagents used was added to the fusion medium, then replenished every 48 h, along with the replacement of the fusion medium. The inhibitors were used at final concentrations of 1.5 mM EGTA (added to fusion medium containing 1.8 mM $CaCl_2$), 30 μM E-64d (diluted from a stock solution of 15 mM in DMSO), 15–25 μM calpeptin (diluted from a stock solution of 10 mM in DMSO [14]), or 0.04–0.08 mg/ml of medium of recombinant calpastatin. For the estimation of the extent of fusion, the cells were washed, fixed and stained with Giemsa. The percent of fused cells was estimated by counting nuclei in myotubes/total of 400–500 nuclei.

2.2. Preparation of cell extracts

The cells in the Petri dishes were washed with PBS, then harvested with PBS containing 5 mM

EDTA. Cells were centrifuged, then lysed in a solution containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2.5 mM EGTA, 0.1 mM *p*-aminoethylbenzenesulfonyl fluoride (AEBSF) and 10 μg of aprotonin/ml solution. Samples were kept in ice for 30 min, then centrifuged at $8000 \times g$. Protein concentration in the supernatants was determined by the method of Lowry et al. [23]. Aliquots of supernatants were mixed with Laemmli sample buffer, heated and proteins separated by SDS-PAGE. Some gels were then stained with Coomassie blue. Other gels were analysed by immunoblotting.

2.3. Effects of calpain added to cell lysates

Cells were grown in Waymouth medium until half confluence, then harvested and lysed as described above. Aliquots of lysates were mixed with a buffer solution of 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.1 mM AEBSF (one volume of lysate containing 50 μg of myoblast protein mixed with four volumes of buffer). The samples were incubated for 15 min at 30°C in the presence of 2.0 mM $CaCl_2$, 0.5 mM dithiothreitol (DTT), with and without added calpain (0.5–1.0 units of calpain purified from human erythrocytes [22]) and calpastatin (1.0–2.0 units of calpastatin purified from human erythrocytes [22]). The samples were then mixed with sample buffer, as described above, electrophoresed, some gels stained with Coomassie blue and others analysed by immunoblotting.

2.4. Immunoblotting procedures

SDS-PAGE was carried out according to standard procedures, using 10% acrylamide. Samples containing 40 μg of myoblast proteins were electrophoresed, then transferred to nitrocellulose membranes (Gelman). Blocking was carried out in a solution containing 20 mM Tris–HCl, 150 mM NaCl, 5% low fat dry milk, 0.05% Tween-20 and 0.02% sodium azide. The membranes were then incubated overnight with the primary antibodies. Antibodies used were: antitalin monoclonal antibody against the 230 kDa whole molecule (Sigma), diluted 1:4000; antitalin polyclonal antibody against the 200 kDa calpain-generated talin fragment (Ref. [24], a gift from Dr. M. Inomata, Tokyo Metropolitan Institute of Gerontology, Japan), diluted 1:300; antiintegrin polyclonal antibody against the 39 amino acids at the C-terminal cytoplasmic domain of integrin β1 (a gift from Dr. R. Hynes, M.I.T., Boston, USA), diluted 1:1000; antitropomyosin and anti β-tubulin monoclonal antibodies (Sigma, USA), diluted 1:2000; anti α-actinin polyclonal antibody (Bio Makor), diluted 1:1000. Following incubation with the primary antibodies, the membranes were washed four times with the blocking buffer (lacking azide) and incubated for 1 h with the appropriate peroxidase-conjugated secondary antibodies [rabbit anti-mouse antibody for the monoclonal antibodies (Amersham), diluted 1:20 000 and goat anti-rabbit antibody for the primary polyclonal antibodies (Sigma), diluted 1:60 000]. Membranes were then washed exhaustively in 20 mM Tris–HCl, 150 mM NaCl, 0.05% Tween-20. Detection of bands was carried out with the ECL immunoblotting detection system (ECL, Amersham).

3. Results

3.1. Effects of inhibitors on myoblast growth and myoblast fusion

Myoblasts, induced to differentiate by changing the growth medium to fusion medium, became confluent at about 24–36 h after the change of medium. Alignment of myoblasts and start of fusion was observed at about 72–96 h, followed by fusion to multinucleated myotubes. About 60–80% of the myoblasts were fused at 120–144 h under the experimental conditions used, as previously described [17] (Fig. 1). Calpastatin inhibited myoblast fusion, with about 5–10% of cell fusion observed following the addition of 0.05 mg of recombinant calpastatin/ml of medium (Fig. 1). About 5% of fusion was obtained by using 30 μM of E-64d (Fig. 1) or 25 μM calpeptin (cell penetrating cysteine protease inhibitors). EGTA inhibited fusion to a similar extent and, under the conditions used, did not affect the cell viability and allowed cell division to continue to confluency (not shown).

3.2. Degradation of membrane proteins in fusing myoblasts and effects of inhibitors

Cell extracts were prepared from myoblasts cultured in growth medium and from cultured cells following the change to fusion medium, in the presence and absence of inhibitors. Aliquots of myoblast extracts were electrophoresed, stained by Coomassie blue or analysed by immunoblotting, as described in Section 2. No gross changes in the overall protein profiles (Coomassie blue staining) were observed in the myoblasts during differentiation and fusion and the profiles of the inhibited, non-fusing myoblasts were similar to those of control cells (Fig. 2A). Immmunoblotting analysis showed degradation of some proteins in myoblasts undergoing fusion. β-Tropomyosin was found to be degraded to a fraction of lower molecular weight (Fig. 2B). Similarly, a partial talin degradation to lower molecular weight fraction was observed in the myoblasts undergoing fusion, as shown by antibody which recognizes both undegraded and degraded talin (Fig. 2C). In addition, using an antibody which selectively recognizes talin fragment generated by calpain-induced talin degradation [24], a degraded talin fraction was identified in the fusing myoblasts (Fig. 2C). Degradation of $\beta 1$ integrin was also observed in these cells (Fig. 2D). No degradation of these proteins occurred in myoblasts prevented from fusing by any of the inhibitors used (Fig. 2B,C,D). On the other hand, α-tropomyosin (Fig. 2B), tubulin (Fig. 2E) and α-actinin (Fig. 2F) did not appear to be degraded in myoblasts undergoing fusion, nor were there any effects of the inhibitors on the appearance of these proteins.

3.3. Effects of calpain and of calpastatin added to myoblast lysates

Myoblast lysates, prepared from proliferating myoblasts (myoblasts cultured in growth medium), were incubated in the presence of Ca^{2+} and DTT, with and without added calpain and calpastatin, followed by SDS-PAGE and immunoblotting, as described in Section 2. As shown by Coomassie blue staining, incubation of myoblast lysate with added calpain resulted in the degradation of some protein fractions, notably in the region of high molecular weight protein fractions above and below 190 kDa, around 100 kDa as well as in the region of about 40–35 kDa fractions. The addition of calpastatin in excess to the added calpain led to inhibition of the protein degradation (Fig. 3A). Myoblast tropomyosin was degraded in these lysates by the added calpain, with β-tropomyosin more susceptible than α-tropomyosin to degradation; the degradation was inhibited by the addition of excess calpastatin (Fig. 3B). Similarly, talin was degraded by calpain, with the degraded talin fragment identified by the antibody specific against talin degraded by calpain [24]; no degradation of talin occurred in the presence of calpastatin (Fig. 3C). Integrin was

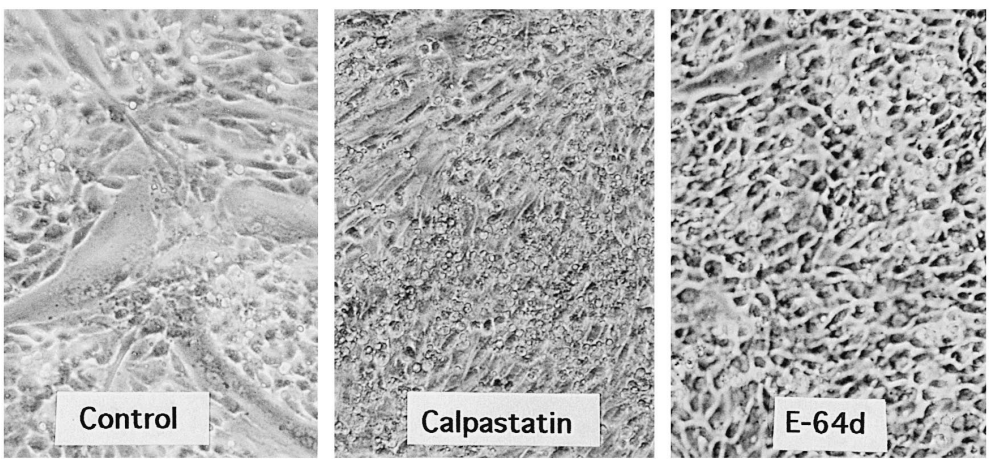

Fig. 1. Phase-contrast micrographs of rat L8 myoblasts. Myoblasts shown at 120 h after change of growth medium to fusion medium without and with the addition of 0.05 mg recombinant calpastatin/ml of medium or with 30 μM E-64d. The inhibitors were replaced every 2 days, along with the replacement of fusion medium (for details, see Section 2).

also degraded by calpain and the degradation was inhibited by added calpastatin (Fig. 3D). In contrast, β-tubulin was not degraded under these conditions (Fig. 3E). α-Actinin in myoblast lysate was also not degraded by calpain (not shown). Thus, the sensitivity of the proteins in lysates of proliferating my-

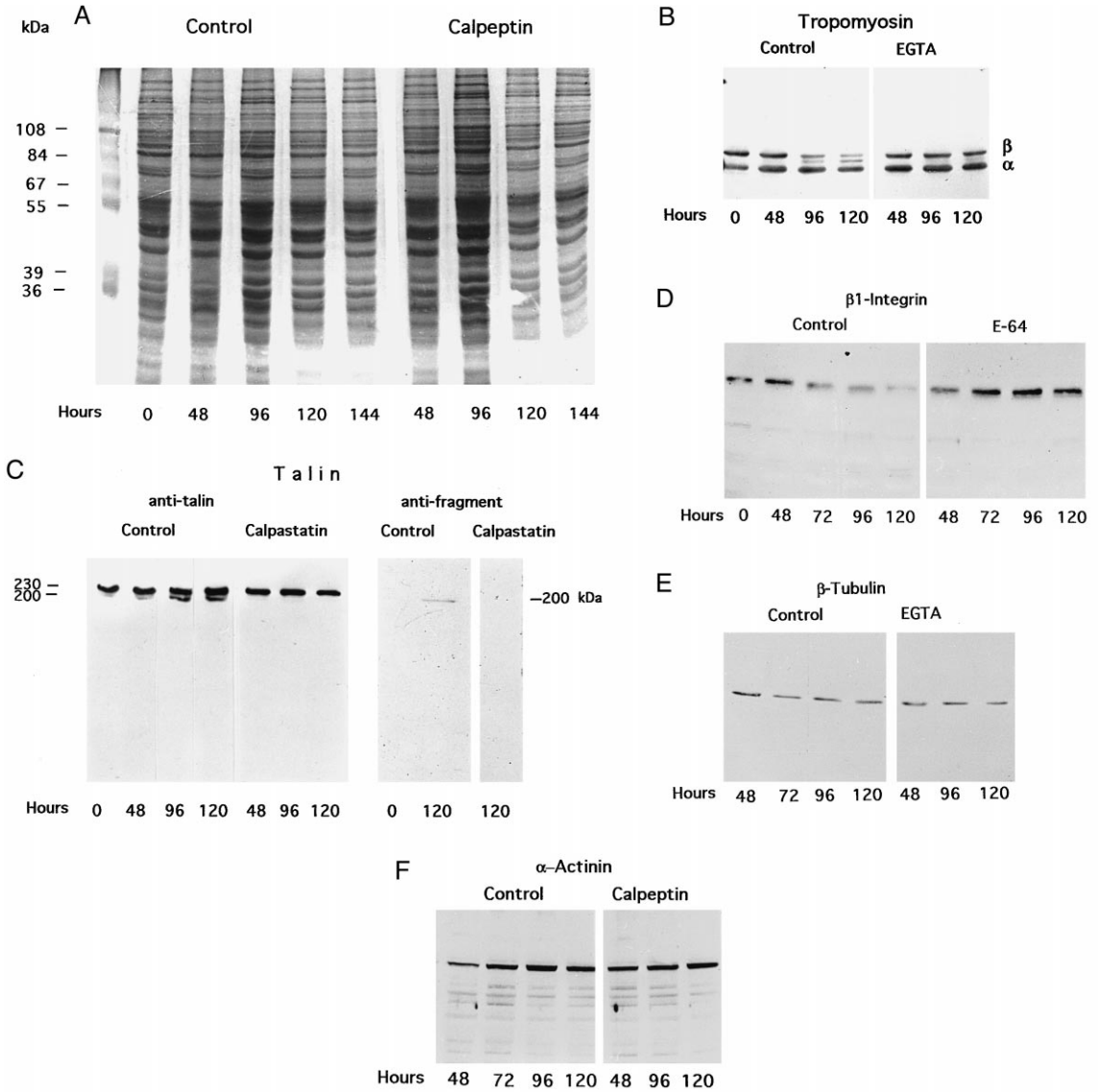

Fig. 2. SDS-PAGE and immunoblotting of myoblast proteins (for details, see Section 2). (A) Coomassie blue stained profiles of myoblast proteins. Myoblast extracts were prepared at the time of change of growth medium to fusion medium (0 h), and at 48, 96, 120 and 144 h; cell extracts were prepared of control, fusing cells and of non-fusing myoblasts inhibited by 20 μM calpeptin. (B) Immunoblotting for the identification of α- and β-tropomyosin in control, fusing myoblasts and in non-fusing cells inhibited by 1.5 mM EGTA. (C) Immunoblotting for the identification of talin in control, fusing myoblasts and in non-fusing cells inhibited by 0.05 mg recombinant calpastatin/ml of medium. Left, immunoblotting with anti-talin antibody, which recognizes undegraded and degraded talin; right, immunoblotting with antibody specific to calpain-induced degradation of talin. (D) Immunoblotting for the identification of β1 integrin in control, fusing myoblasts and in non-fusing cells inhibited by 30 μM E-64d. (E) Immunoblotting for the identification of β-tubulin in control, fusing myoblasts and in non-fusing cells inhibited by 1.5 mM EGTA. (F) Immunoblotting for the identification of α-actinin in control, fusing myoblasts and in non-fusing cells inhibited by 20 μM calpeptin.

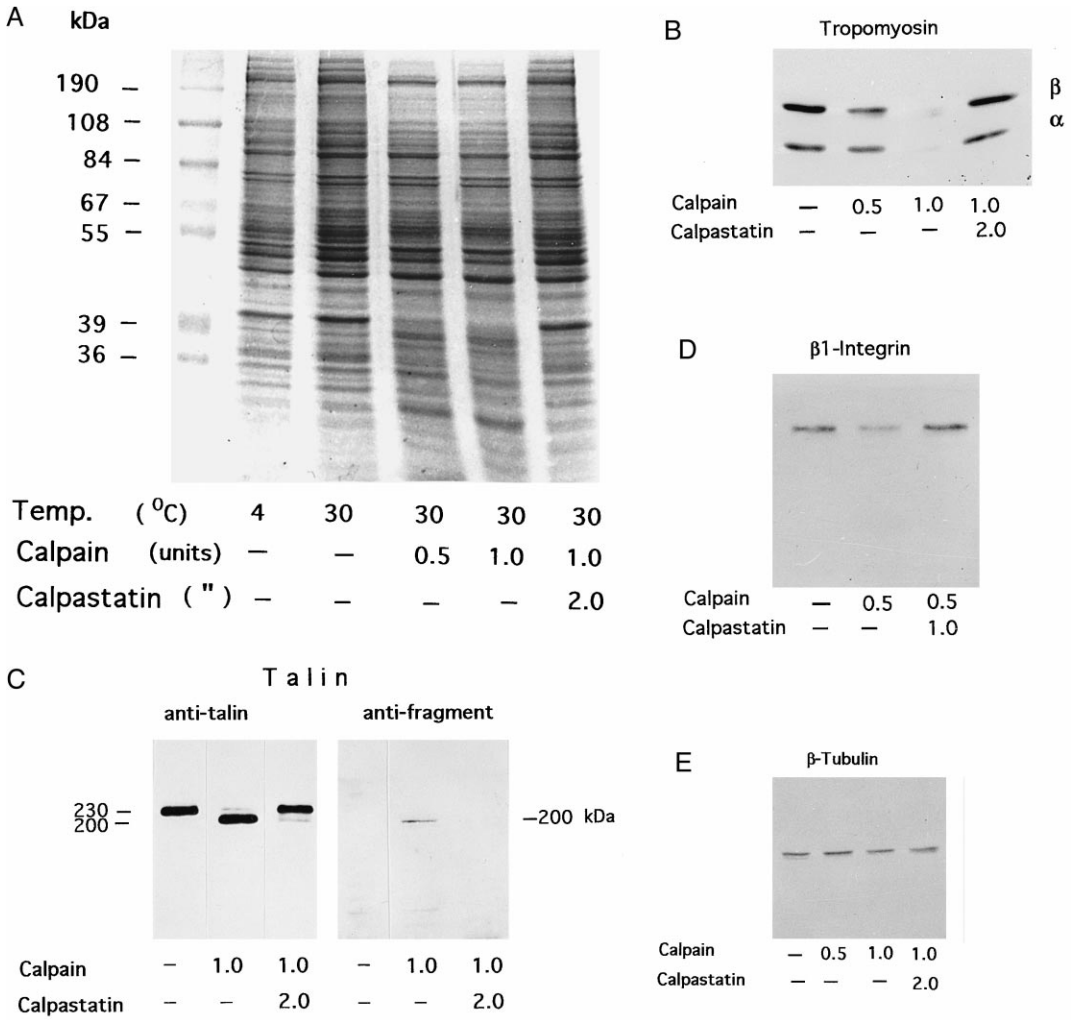

Fig. 3. SDS-PAGE and immunoblotting of lysates of proliferating myoblasts. The lysates were treated with and without calpain and calpastatin (for details, see Section 2). (A) Coomassie blue stained profiles of proteins in myoblast lysates. (B) Immunoblotting for the identification of α- and β-tropomyosin in the control and treated lysates. (C) Immunoblotting for the identification of talin and calpain-induced talin degradation. Left, immunoblotting with anti-talin antibody, which recognizes undegraded and degraded talin; right, immunoblotting with antibody specific to calpain-induced degradation of talin. (D) Immunoblotting for the identification of $\beta 1$ integrin in the control and treated lysates. (E) Immunoblotting for the identification of β-tubulin in the control and treated lysates.

oblasts to degradation by added calpain appeared to parallel the behavior of these proteins in the intact myoblasts undergoing fusion.

4. Discussion

Fusing myoblasts have been shown to temporarily develop numerous lacunae, which appear to be regions of the membrane lipid bilayer devoid of some surface proteins [7]. The formation of the lacunae is not due to a drastic loss of membrane proteins, but to destabilization and disorganization of the myoblast skeletal network during fusion [7] and may correspond to the lipid-rich domains, relatively free of proteins, observed in fusing myoblasts [5,7]. Cell shape stabilization, interaction with other cells and with extracellular matrix (ECM) are controlled by associations among the cytoskeletal proteins and by proteins connecting the cytoskeleton to the plasma membrane [25]. Destabilization of the skeletal network in the fusing myoblasts would result from

disrupting the linkages between the cytoskeleton and the plasma membrane.

We show here that integrin $\beta 1$ subunit, talin and β-tropomyosin are degraded in the fusing myoblasts, whereas α-actinin, β-tubulin and α-tropomyosin are not. Integrins are a major family of heterodimeric cell surface receptors, composed of various α and β subunits, that mediate interactions of cells with the ECM and with other cells [25–27]. Integrins mediate bidirectional transfer of information across the plasma membrane by linking extracellular proteins to cytoskeleton proteins and actin filaments, with some shown to bind to cytoskeletal proteins through the integrin $\beta 1$ cytoplasmic domain [25–27]. Degradation of the cytoplasmic domain of integrin $\beta 1$ subunit may thus modulate the attachment of the cell to the ECM and the organization of the cytoskeleton.

Disruption of membrane–cytoskeleton linkages would also result from degradation of linker proteins, such as talin. Talin is required for the stability of the adhesion plaques, where communications between the cytoskeleton and the ECM are maintained, and cleavage of talin is associated with detachment of cultured cells from the ECM [28]. Both talin and α-actinin, in addition to linking actin filaments to integrin, may also bind directly to acidic phospholipids [25]. Disruption of such binding may play a part in the altered phospholipid organization and redistribution of the bilayer phospholipids, a redistribution that contributes to the fusion competency of the myoblasts [29]. In addition to the myoblast proteins studied here, the actin-binding protein filamin and the intermediate filament desmin have also been shown to be degraded in fusing myoblasts [30,31]. The degradation of these proteins and degradation of actin-stabilizing proteins such as β-tropomyosin, would add to the skeletal disorganization and to freeing of membrane domains from the restraints of linkage to the cell cytoskeleton.

The inhibition of protein degradation by cysteine protease inhibitors, including the specific calpain inhibitor calpastatin, is consistent with the notion that calpain is involved in protein degradation in fusing myoblasts. Support for calpain involvement in protein degradation in the fusing myoblasts is also provided by the identification of a calpain-specific talin fragment, using an antibody which selectively recognizes a 200 kDa fragment when talin is digested by calpain [24] and the inhibition of that degradation by calpastatin. The fact that intracellular protein degradation is inhibited by the calpastatin added to intact differentiating myoblasts indicates that calpastatin is being taken up by these cells, though the mechanism for the uptake is not known. Since calpastatin is known to be sensitive to degradation by some enzymes [19,20], it may be taken up as active fragment(s) rather than as a large molecule. The mode of uptake waits further study.

The results shown here indicate that the protein degradation in the fusing myoblasts is not a generalized protein breakdown. The fact that talin is degraded in the fusing myoblasts but α-actinin is not points to the selectivity of protein degradation during myoblast differentiation. Talin is a physiological substrate for calpain during platelet aggregation [24,33] and is the preferred substrate for calpain among the adhesion plaque proteins [32]. Selectivity of protein degradation by calpain has been shown in some cell types [28,32] and has been suggested to be due to an association of the protease with a substrate in specific locations such as the adhesion plaques [28,32]. It should be pointed out that even an excess of calpain added in vitro to cell lysates does not cause a general protein degradation but only degradation of some proteins. Thus, the degradation of the particular proteins observed in the fusing myoblasts but not in the proliferating cells appears not to be due to changes in substrate properties, since their degradation is possible when the endogenous high level of calpastatin in proliferating myoblasts is overcome by excess calpain added to Ca^{2+}-containing lysates of these cells.

Calpain activation, made possible by the diminution in calpastatin in fusing myoblasts [16,17], requires Ca^{2+}. Cytosolic Ca^{2+} has been shown to be elevated during myoblast fusion [34]. Calpain activation is generally considered to be enhanced by its translocation to the membrane and by association with phospholipids [20,35–37]. It is not clear whether μ-calpain or *m*-calpain is mostly involved in myoblast fusion. Since μ-calpain can activate *m*-calpain [38], it is possible that both are involved. Cytoplasmic unilamellar, particle-free vesicles have been described to appear in cultured, fusing myoblasts in close proximity to the plasma membrane [5]. It has been proposed that the interaction of the cytoplasmic vesicles with the plasma membrane initiates the

membrane destabilization and the formation of the fusion domains [5]. Prefusion complexes, consisting of intracellular vesicles, that align and pair across apposed myoblast membranes with vesicles in the other cell, have been shown to form in myoblasts fusing in vivo [9]. The composition of the vesicles is not known at present. It would be of interest to study whether they play a role in protein degradation in the fusing myoblasts. It should also be noted that in addition to being involved in protein degradation, calpain may affect membrane–cytoplasmic interactions indirectly. One such possibility is calpain-induced activation of PKC, leading to phosphorylation of proteins such as the myristoylated alanine-rich protein kinase C substrate (MARCKS) and resulting in the dissociation of the protein from the membrane and altered membrane organization [39,40].

5. Conclusion

Degradation of certain proteins, known to be responsible for the stability of the membrane–skeleton organization and for the interaction of the cell with the ECM, appears to be required for myoblast fusion. Data presented here support the notion that calpain participates in the fusion-associated protein degradation. The participation of calpain in the fusion process does not exclude the involvement of other proteases, such as cathepsin B, in myoblast fusion [41]. In addition to the participation of the calpain–calpastatin system in myoblast fusion, calpain-induced protein degradation, controlled by calpastatin, may be involved in other cell membrane sealing and repair processes [42,43].

References

[1] M.J.O. Wakelam, Biochem. J. 228 (1985) 1–12.
[2] D. Yaffe, in: P.F. Kruse, M.K. Patterson (Eds.), Tissue Culture: Methods and Applications, Academic Press, New York, 1973, pp. 106–114.
[3] T. Bachi, M. Aguet, C. Howe, J. Virol. 11 (1973) 1004–1012.
[4] E.M. Kosower, N.S. Kosower, P. Wegman, Biochim. Biophys. Acta 471 (1977) 311–329.
[5] N. Kalderon, N.B. Gilula, J. Cell Biol. 81 (1979) 411–425.
[6] M. Tavassoli, N.S. Kosower, C. Halverson, A. Makoto, E.M. Kosower, Biochim. Biophys. Acta 601 (1980) 544–558.
[7] A.B. Fulton, J. Prives, S.R. Farmer, S. Penman, J. Cell Biol. 91 (1981) 103–112.
[8] R. Yanagimachi, in: E. Knobil, J. Neill (Eds.), The Physiology of Reproduction, Raven Press, New York, 1988, pp. 135–185.
[9] S.K. Doberstein, R.D. Fetter, A.Y. Mehta, C.S. Goodman, J. Cell Biol. 136 (1997) 1249–1261.
[10] J.E. Schollmeyer, Exp. Cell Res. 162 (1986) 411–422.
[11] N.S. Kosower, T. Glaser, in: R.L. Mellgren, T. Murachi (Eds.), Intracellular Ca^{2+}-dependent Proteolysis, CRC Press, Boca Raton, FL, 1990, pp. 163–180.
[12] M. Hayashi, Y. Saito, S. Kawashima, Biochem. Biophys. Res. Commun. 182 (1992) 939–946.
[13] K.B. Kwak, S.S. Chung, O.M. Kim, M.S. Kang, D.B. Ha, C.H. Chung, Biochim. Biophys. Acta 1175 (1993) 243–249.
[14] C. Ebisui, T. Tsujinaka, Y. Kido, S. Iijima, M. Yano, H. Shibata, T. Tanaka, T. Mori, Biochem. Biol. Int. 32 (1994) 515–521.
[15] P. Cottin, J.J. Brustis, N. Poussard, N. Elamrani, A. Broncard, A. Ducastaing, Biochim. Biophys. Acta 1223 (1994) 170–178.
[16] S. Barnoy, T. Glaser, N.S. Kosower, Biochem. Biophys. Res. Commun. 220 (1996) 933–938.
[17] S. Barnoy, T. Glaser, N.S. Kosower, Biochim. Biophys. Acta 1358 (1997) 181–188.
[18] R.L. Mellgren, FASEB J. 1 (1987) 110–115.
[19] D.E. Croall, G.N. DeMartino, Physiol. Rev. 71 (1991) 813–847.
[20] T.C. Saido, H. Sorimachi, K. Suzuki, FASEB J. 8 (1994) 814–822.
[21] N.S. Kosower, T. Glaser, E.M. Kosower, Proc. Natl. Acad. Sci. U.S.A. 80 (1983) 7542–7546.
[22] T. Glaser, N.S. Kosower, FEBS Lett. 206 (1986) 115–120.
[23] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1951) 265–275.
[24] M. Inomata, M. Hayashi, Y. Ohno-Iwashita, S. Tsubuki, T.C. Saido, S. Kawashima, Arch. Biochem. Biophys. 328 (1996) 129–134.
[25] E.J. Luna, A.L. Hitt, Science 258 (1992) 955–962.
[26] E.A. Clark, J.S. Brugge, Science 268 (1995) 233–238.
[27] S. Dedhar, G.E. Hannigan, Curr. Opin. Cell Biol. 8 (1996) 657–669.
[28] L. Tranqui, M. Block, Exp. Cell Res. 217 (1995) 149–156.
[29] A. Sessions, A.F. Horwitz, Biochim. Biophys. Acta 728 (1983) 103–111.
[30] K.B. Kwak, J.-i. Kambayashi, M.S. Kang, D.B. Ha, C.H. Chung, FEBS Lett. 323 (1993) 151–154.
[31] N. Elamrani, J.J. Brustis, N. Dourdin, D. Balcerzak, S. Poussard, P. Cottin, A. Ducastaing, Biol. Cell. 85 (1994) 177–183.
[32] M.C. Beckerle, K. Burridge, G.N. DeMartino, D.E. Croall, Cell 51 (1987) 569–577.
[33] J.E.B. Fox, D.E. Goll, C.C. Reynolds, D.R. Phillips, J. Biol. Chem. 260 (1985) 1060–1066.

[34] B. Constantin, C. Cognard, G. Raymond, Cell Calcium 19 (1996) 365–374.
[35] S. Pontremoli, F. Salamino, B. Sparatore, M. Michetti, O. Sacco, E. Melloni, Biochim. Biophys. Acta 831 (1985) 335–339.
[36] D.E. Goll, W.C. Kleese, A. Okitaki, T. Kumamoto, J. Cong, H.P. Kapprell, in: R.L. Mellgren, T. Murachi (Eds.), Intracellular Ca^{2+}-dependent Proteolysis, CRC Press, Boca Raton, FL, 1990, pp. 3–24.
[37] M. Inomata, M. Hayashi, M. Nakamura, Y. Saito, S. Kawashima, J. Biol. Chem. 264 (1989) 18838–18843.
[38] P. Tompa, A. Baki, E. Schad, P. Friedrich, J. Biol. Chem. 271 (1996) 33161–33164.
[39] J.H. Hartwig, M. Thelen, A. Rosen, P.A. Janmey, A.C. Nairn, A. Aderem, Nature 356 (1992) 618–622.
[40] S. Swierczynski, J. Blackshear, J. Biol. Chem. 271 (1996) 23424–23430.
[41] J.A. Gogos, R. Thompson, W. Lowry, B.F. Sloane, H. Weintraub, M. Horwitz, J. Cell Biol. 134 (1996) 837–847.
[42] P.L. McNeil, R.A. Steinhardt, J. Cell Biol. 137 (1997) 1–4.
[43] C.M. Godell, M.E. Smyers, C.S. Eddleman, M.L. Ballinger, H.M. Fishman, G.D. Bittner, Proc. Natl. Acad. Sci. U.S.A. 94 (1997) 4751–4756.